SUBSTRATE SPECIFICITY OF AMINE OXIDASE

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The tyramine oxidase activity of liver extracts found by Hare (1), the aliphatic amine oxidase activity of brain, kidney, and liver extracts observed by Pugh and Quastel (2), and the adrenalin oxidase activity of similar extracts noted by Blaschko, Richter, and Schlossmann (3) were brought under a common enzyme view-point by the latter authors. They were able to show (4) that extracts of brain, intestine, kidney, and liver from a number of mammals or representatives of the birds, reptiles, amphibians, and fishes all acted to absorb oxygen in the presence of several amine substrates. Hare (1) had shown that tyramine and phenethylamine form ammonia in the course of such oxidations, and Richter (5) showed that an ethylamino and a dimethylamino compound, as well as a number of methylamino and amino compounds, all yield the corresponding alkylamines or ammonia in the enzymic oxidation.

The conclusion that the demonstrated variety of such enzymic activity can be ascribed to the presence of only a single type of amine oxidase was dependent in large part on observations that the relative activities of a preparation from one source on a series of substrates bear some relation to the relative activities exhibited by a preparation from another source. Further evidence depended upon the action of certain amines as inhibitors and apparent competition between substrates when two oxidizable substrates are present in the system. The degree to which the relative activities of different enzyme preparations were constant in a series of substrates was not good in the data reported, and the fact that Hare (1) had not been able to note activity of the liver preparations she used upon adrenalin as the substrate appeared to require special explanations.

After the present work was begun, it became obvious that relative to activity based on use of tyramine as substrate activity differences are notable on other substrates with liver extracts derived from different animal species. It appeared very desirable to attempt purification of the crude extracts used to see whether during the process changes would result in the relative activity when tested on a series of substrates. Another object of purification was to prepare material suitable for trial injection into

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animals made hypertensive, an experiment which was carried out in idea by Schroeder (6) but with doubtful adequate activity of the enzyme used.

The degree of purification achieved after very considerable work was not very great, but an effective and rapid separation from readily dialyzable substances and materials that are soluble at pH 6 was developed. The activity of such partially purified preparations was determined with considerable precision on a number of substrates to determine the effect on the oxidative reaction of small but important changes in the structure of aliphatic, phenylaliphatic, and substituted phenylaliphatic amine substrates. Such observations form a basis for consideration of the deamination of these various amines by the liver when they naturally occur or are introduced in the body, an idea that has been partially developed for some phenylaliphatic amines by Beyer and Lee (7). The kinetic equilibrium constants for amine oxidase and its amine substrates here reported may be expected to form bases for ideas of the combination of these same amines with physiologically active receptor mechanisms.

Species Variance of Amine Oxidase—Crude enzyme preparations were made by grinding livers of freshly killed animals with 4 ml. per gm. of 0.2 M phosphate buffer, pH 7.0, then screening to remove fiber, and centrifuging in a supercentrifuge. The test system consisted of 2.0 ml. of the enzyme solution, 0.3 ml. of 0.1 M sodium cyanide, and 0.2 ml. of a 0.1 M solution of amine salt (chloride or sulfate). Oxygen uptake was measured at 30° with the usual Warburg technique, with readings every 5 minutes. From plots of the data, maximum rates of oxygen uptake were determined and calculated into terms of per cent based on the initial rate of oxygen uptake of phenethylamine. The data of Table I are averages of two sets of experiments for each animal species.

The extent to which the data of Table I are comparable to the data given by Blaschko, Richter, and Schlossmann (4) for certain of these substrates when acted on by guinea pig or other liver preparation is impossible to determine. The observations here are corrected for the oxygen uptake of the blank, which was very small in all cases owing to working promptly with fresh extracts only. Aside from minor differences in the pH and temperature of observation, wholly different comparative values may result with certain types of substrate by taking only initial rates as the basis of observation instead of periods of as long as 1 hour. Most notably with the aliphatic amines, the rate of oxygen uptake decreases with time. With the substrate concentrations used, the falling off of rate with any of the substrates appears unrelated to disappearance of substrate or of enzyme, and is probably an inhibition of enzyme by reaction products, as found by Hare (1). Whatever the cause, it is apparent that maximum rates of oxidation are most likely to give a clearer picture of the substrate specificity (see Fig. 1).

Purification of Amine Oxidase—Preparation of a cell-free solution of the enzyme from rabbit liver extract by adsorption on kaolin and elution of the product was mentioned by Hare (1). Kohn (8) found the enzyme to be precipitated from pig liver extract at a reaction acid to methyl red and the insoluble material with which it was associated to be resuspendable at pH 7 to 8. Amine oxidase activity has only been observed in the present

TABLE I

Liver Extract Oxidations; Maximum Oxidation Rates (Per Cent) Relative to Phenethylamine

At 30° with 0.008 M amine substrates in phosphate buffer, pH 7.0.

	Rabbit	Guinea pig	Cat	Cattle
Ethylamine	0	0	20	5
Butylamine	50	100	90	110
Amylamine	110	140	110	85
Hexylamine	120	80	90	85
Heptylamine	130	70	100	85
Benzylamine	30	10	100	130
Phenethylamine	100	100	100	100
Phenpropylamine	110	30	90	105
Phenethanolamine	30	30	55	5
Phenethylmethylamine	105	120	105	65
3-Hydroxyphenethylamine	70	190	110	95
4-Hydroxyphenethylamine (tyramine)	90	200	120	130
4-Hydroxyphenethylmethylamine	65	160	80	85
4-Hydroxyphenethanolmethylamine (synephrine)	25	70	40	5
4-Hydroxyphenethyldimethylamine (hordenine)	30	10	45	80
3,4-Dihydroxyphenethylamine (hydroxytyramine)	65	200	80	85
3,4-Dihydroxyphenethylmethylamine (epinine)	65	180	30	65
3,4-Dihydroxyphenethanolamine (arterenol)	25	40	20	5
3,4-Dihydroxyphenethanolmethylamine (*dl*-epinephrine)	15	40	20	5
l-3,4-Dihydroxyphenethanolmethylamine (epinephrine)	25	40	20	5

work in turbid "solutions," and in general the activity and turbidity of freshly prepared extracts seemed to be proportional. With salts present, the amine oxidase activity cannot be centrifuged out if the pH is 7 or higher, and only partially at lower pH values. If salts are removed by dialysis and the pH adjusted to 6.0, a flocculent precipitate separates that contains almost all of the amine oxidase activity present. Washing this

precipitate with water adjusted to pH 6.0 removes a considerable amount of inert material with only a small loss of enzyme activity; then the precipitate can be redispersed by addition of salts or adjustment to pH 7 or higher so that it cannot be removed by centrifugation in a Sharples machine. Storage of the precipitate obtained at pH 6.0 at that acidity is apt to result in a decrease in "solubility" in salt solutions or at higher pH values, but without considerable change in activity, showing that activity is observable with definitely insoluble particles that may be removed by centrifugation or filtration.

The marked instability of amine oxidase outside of the pH range of 5.5 to 8.5 greatly limits the purification procedures that may be used. Addition of but 30 per cent alcohol completely inactivates the enzyme in a short

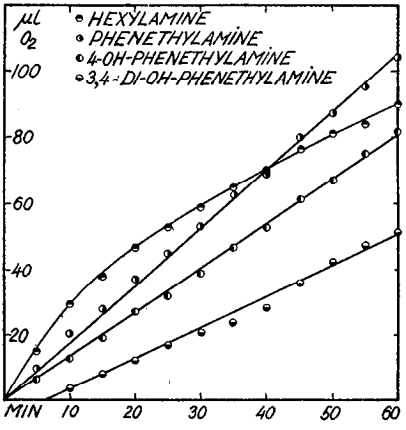

FIG. 1. Oxygen uptake in microliters against time in minutes for different substrates. At 30° with 2.0 ml. of 1:4 rabbit liver extract with 0.2 M phosphate buffer, pH 7.0, 0.3 ml. of 0.1 M sodium cyanide, and 0.2 ml. of 0.1 M amine salt solution.

time, and while precipitation of the activity may also be accomplished with addition of ammonium sulfate to one-third saturation, such salting-out is not a useful purification procedure. Such salting-out results in a considerable loss (25 to 40 per cent) of activity and does not offer any advantage over isoelectric precipitation, particularly since the high density of the salting-out solution makes difficult the centrifugation of the precipitate. Procedures involving adsorption of the activity on such agents as kaolin and tricalcium phosphate were tried under various conditions, but were not practicable because of unsatisfactory recovery of the activity from the adsorbates.

Most of the purification work from which the above conclusions were drawn was carried out with cattle liver extracts made by grinding the fresh

tissue with some ice water in a Waring blendor at high speed, then making the mixture to 500 volumes per cent of the tissue weight. For later work, because of the importance of using fresh tissue for each preparation, rabbit liver extracts were chiefly used and the work reported in this paper without mention of source was carried out only with enzyme preparations from rabbit liver.

As a standard in the purification work, a unit of amine oxidase activity was taken as the amount of enzyme which will catalyze the uptake of 1 microliter of O_2 per minute at 30° and pH 7.0 in the presence of 0.008 M tyramine. The protein content of the preparations was readily determined by pipetting 10 ml. into 20 ml. of 20 per cent trichloroacetic acid, adding 100 mg. of diatomaceous earth filter aid, filtering after 10 minutes, washing the precipitate with water, then ether, and drying at 80° before weighing. The stability of amine oxidase preparations is fair when kept at pH 7 in a refrigerator at 0–5°, and only small losses in activity were usually observed during as much as a week of storage, but an increasing ability of the preparation to absorb oxygen without any substrate addition may become considerable after only a few days and no data are reported for preparations over 4 days old. The change in the preparation on storage appears to be due to other enzymes, as the addition of 1:10,000 to 1:20,000 phenylmercuric acetate served to insure sterility without any effect on amine oxidase activity or its keeping qualities.

The purified amine oxidase preparations used for study of substrate specificity and the kinetics of the oxidation of certain amines were made as follows:

A liver from a rabbit immediately after it was killed (70 to 90 gm.) was ground with ice and water in a blendor; then the volume was made up to 500 ml. and the pH adjusted to 8.0 with a glass electrode. After fiber was screened out, the mixture was passed twice through a Sharples supercentrifuge and then placed in Visking tubing and dialyzed for 20 hours into 10 liters of water within a refrigerator. Adjustment of the dialyzed solution to pH 6.0 with acetic acid yielded a flocculent precipitate which was centrifuged out. The precipitate was resuspended in 1 liter of distilled water and the pH adjusted to 6.0, the insoluble solids centrifuged off, and the centrifugate discarded. The solids were then resuspended in water with addition of some concentrated phosphate buffer solution and some 1:1000 phenylmercuric acetate solution and exact adjustment of pH, so that the final solution was 250 ml. in volume and contained 0.1 M sodium phosphate buffer of pH 7.0 and was 1:10,000 in phenylmercuric acetate. This suspension which was used in the enzyme experiments was a pink-red, turbid fluid.

Following a preparation through this procedure with regard to protein

content and enzyme activity showed 9.8 gm. of protein and 440 enzyme units in the original dialysate. The supernatant liquid from the precipitation at pH 6.0 contained 4.7 gm. of protein and about 35 enzyme units, and the wash solution contained 0.7 gm. of protein and about 25 enzyme units. The final enzyme solution contained 3.8 gm. of protein and 280 enzyme units. Thus, the handling losses in this preparation amounted to about 6 per cent of the protein and about 20 per cent of the enzyme activity, but the loss was justified by the gain in activity per unit of protein and other total solid content.

Combined Influence of pH and Substrate on Activity—The effect of pH on tyramine oxidation with amine oxidase was studied by Hare (1). The enzyme and substrate were mixed, the pH adjusted to different values, and

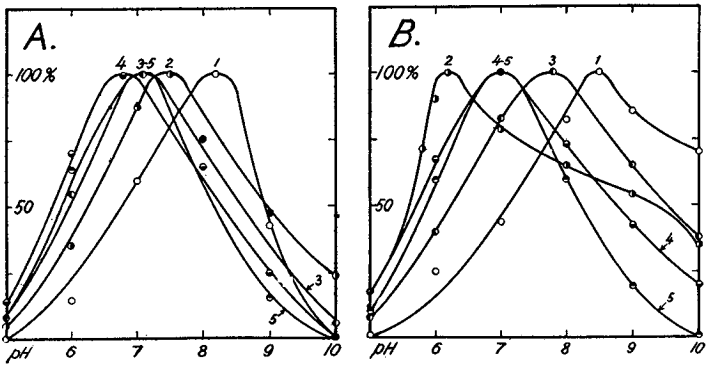

FIG. 2. Relative rates of oxygen uptake against pH for different substrates. The rates are calculated as per cent of rate of each compound at optimum pH. *A*, Curve 1 butylamine, Curve 2 amylamine, Curve 3 hexylamine, Curve 4 heptylamine, Curve 5 octylamine; *B*, Curve 1 phenmethylamine, Curve 2 phenethylamine, Curve 3 phenpropylamine, Curve 4 phenbutylamine, Curve 5 phenamylamine.

the oxygen uptake of the mixture then determined. The experiments indicated a pH optimum at about 9 to 10, with rapid decrease in activity at both higher and lower pH values; so that oxygen uptake was only about half of the maximum at pH 7. Interpretation of the decrease above pH 10 is complicated in such experiments, for Hare showed that notable destruction of the enzyme activity occurs within 5 minutes at such high alkalinities.

The pH and activity relationship was studied in the present work with a number of different substrates to determine to what extent it would vary within a series of amines. The alkylamines afford a series of compounds that are ionized in water solution to about the same extent, according to available data (see Alles (9)). These compounds only show a variation in pK_b at 25° from 3.34 to 3.59. As may be noted from Fig. 2, the

optimum pH for these compounds extends from about 8.1 for butylamine through a minimum of about 6.9 for heptylamine, and is around 7.1 to 7.2 for octyl and higher amines.

Results obtained with a series of phenalkylamines (ω-phenylalkylamines) are also shown in Fig. 2, *B* and are of particular interest because good determinations of their ionization constants in water solutions are available from the work of Carothers, Bickford, and Hurwitz (10). Their pK_b values vary from phenmethylamine 4.63, phenethylamine 4.17, phenpropylamine 3.80, phenbutylamine 3.60, to phenamylamine 3.51. The ionization constants of the latter two amines are within the range for

TABLE II

Maximum Oxidation Rates (Per Cent) Relative to Phenethylamine

At 30° with 0.008 M amine substrates in 0.1 M phosphate, pH 7.0.

Enzyme Preparation		A	B	C	Average
Straight chain compounds	Methylamine	0	0	0	0
	Ethylamine	0	0	0	0
	Propylamine	0	0	0	0
	Butylamine	36	62	55	51
	Amylamine	101	102	116	106
	Hexylamine	115	104	123	114
	Heptylamine	129	122	130	127
	Octylamine	133	145	141	140
Branched chain compounds	β-Methylpropylamine (isobutylamine)	9	8	13	10
	β-Methylbutylamine	71	82	95	83
	γ-Methylbutylamine (isoamylamine)	86	100	113	100
	γ-Methylamylamine	87	92	106	95
	δ-Methylhexylamine (isohexylamine)	98	100	113	103

alkylamines, indicating the effect of the phenyl group to be lost. The weakest of these bases, phenmethylamine, requires the most strongly basic environment for maximum activity, but phenethylamine falls out of any regular series variation in that its maximum activity is at the lowest pH of any of the series. It should be noted that the plot of Fig. 2 is made to indicate most clearly the pH variation for each compound by assigning 100 per cent value to the maximum rate at the pH optimum for each amine separately, and does not indicate that the maximum oxidation rate for the several compounds is the same at their optimum pH. The relative oxidation rates for the same compounds at pH 7.0 alone are given in Tables II and IV.

Aliphatic Primary Amines As Substrates—The oxidation system consisted of 2.0 ml. of purified amine oxidase preparation in 0.1 M phosphate of pH 7.0 and 0.3 ml. of water, to which was added at zero time 0.2 ml. of 0.1 M amine salt solution. The amine salts were mostly sulfates, though chlorides were used on occasion without notable anion effect. Readings were taken every 2.5 minutes to determine accurately the form of the O_2 uptake against time relationship, and the rates recorded in the tables are maximum rates, often largely determined from the first few readings. A control of the enzyme preparation without added amine was run at the same time as was a comparison standard containing 0.2 ml. of 0.1 M phenethylamine. Results were discarded if more than a negligible oxygen uptake was noted in the control during the course of the experiment. By repeating such experiments and calculating on the basis of the simultaneous uptake rate observed for phenethylamine average values were obtained as shown in Table II.

The compounds of Table II are all primary carbinamines, and it is to be noted that the branched chain compounds were somewhat less actively oxidized than the straight chain compound of corresponding total number of carbon atoms. The rate of oxidation increases among the series of compounds as the branching of the chain is further removed from the amino group.

A series of secondary carbinamines in which an α-methyl group was introduced into the series of normal alkylamines was particularly studied. All of the following α-methylalkylamines were found to be completely unoxidizable under the same conditions as described for the testing of the primary carbinamines: α-methylethylamine (isopropylamine), α-methylpropylamine (*sec*-butylamine), α-methylbutylamine (*sec*-amylamine), α-methylamylamine, α-methylhexylamine, α-methylheptylamine, α-methyloctylamine, α-methylnonylamine.

A corresponding series of tertiary carbinamines in which two α-methyl groups were introduced into the series of normal alkylamines was also studied. All of the following α,α-dimethylalkylamines were completely unoxidizable under the same conditions: α,α-dimethylethylamine (*tert*-butylamine), α,α-dimethylpropylamine (*tert*-amylamine), α,α-dimethylbutylamine, α,α-dimethylamylamine, α,α-dimethylhexylamine, α,α-dimethylheptylamine.

Although Bhagvat, Blaschko, and Richter (11) noted no oxidation of cadaverine or putrescine by crude extracts of guinea pig intestine and liver, it appeared desirable to extend the observations to the aliphatic diamines more generally. Those studied were all primary carbinamines and the higher members of the series might be expected to behave more like monoamines, since their groups are too far apart to transmit effects along their

carbon chains. The following compounds were completely unoxidized by amine oxidase under the conditions described for the primary carbin-monoamines: ethylenediamine, trimethylenediamine, tetramethylenediamine (putrescine), pentamethylenediamine (cadaverine), hexamethylenediamine, heptamethylenediamine, octamethylenediamine.

One additional point seemed worthy of special investigation with the primary carbinamines as substrates. In comparisons made by Blaschko, Richter, and Schlossmann (4) of the relative effects of crude liver extracts of guinea pig, rat, or pig on various substrates, it is notable that heptylamine is less oxidized in an hour than is isoamylamine or phenethylamine. A like situation was noted in our experiments with guinea pig, cat, and cattle liver extracts, as shown by the data of our Table I in which maximum oxidation rates only are considered. The results with rabbit liver extracts are dissimilar, and it is even more notable that among the series of the aliphatic primary carbinamines heptylamine is the most actively oxidized by rabbit liver extract, while with guinea pig, cat, and cattle liver extracts

TABLE III

Maximum Oxidation Rates (Per Cent) Relative to Phenethylamine with Purified Cattle Liver Amine Oxidase Preparation

At 30° with 0.008 M amine substrates in 0.1 M phosphate, pH 7.0.

Methylamine	7	Amylamine	91
Ethylamine	8	Hexylamine	91
Propylamine	75	Heptylamine	89
Butylamine	117	Octylamine	75

one of the lower members of the series represents a maximum for the series. Further investigation of this phenomenon was carried out by purifying a cattle liver extract by following the procedure described for purified preparations of rabbit liver amine oxidase, then carrying out oxidation studies in the same manner as were those described in Table II. The results are shown in Table III. In contrast to the results with amine oxidase preparations from rabbit liver, there is clearly a maximum of activity among this series of amines, and the three lowest members of the series are oxidizable, propylamine being quite notably oxidized, whereas with rabbit liver preparations it is not at all.

N-Methyl Derivatives of Aliphatic Amines As Substrates—Although Blaschko, Richter, and Schlossmann (4) found with extracts of guinea pig intestine and liver that the symmetrical dialkylamines and trialkylamines were not oxidized, it seemed important to study a number of alkylmethylamines and alkyldimethylamines in comparison with the alkyl primary amines. The oxidation system and conditions were kept as for the experi-

ments of Table II; so that the oxidation rates observed with the alkylamines in these experiments were assigned the average values as there given and the oxidation rates of the N-methyl and N-dimethyl derivatives calculated on this basis. The alkyltrimethylammoniums were included in this grouping of compounds, for the only previously investigated compound

TABLE IV

Maximum Oxidation Rates (Per Cent) Relative to Phenethylamine

At 30° with 0.008 M amine substrates in 0.1 M phosphate, pH 7.0.

Butylamine	51	Hexylamine	114
Butylmethylamine	27	Hexylmethylamine	46
Butyldimethylamine	0	Hexyldimethylamine	5
Butyltrimethylammonium	0	Hexyltrimethylammonium	0
Amylamine	106	Heptylamine	127
Amylmethylamine	57	Heptylmethylamine	70
Amyldimethylamine	0	Heptyldimethylamine	15
Amyltrimethylammonium	0	Heptyltrimethylammonium	0

TABLE V

Maximum Oxidation Rates (Per Cent) Relative to Phenethylamine

At 30° with 0.008 M amine substrates in 0.1 M phosphate, pH 7.0.

Phenmethylamine (benzylamine)	36	Phenpropylamine (γ-phenylpropylamine)	109
Phenethylamine (β-phenylethylamine)	100	Phenbutylamine (δ-phenylbutylamine)	118
β-Methylphenethylamine (β-phenylpropylamine)	67	Phenamylamine (ϵ-phenylamylamine)	115
Phenethylmethylamine	91	Phenethylethylamine	23
Phenethyldimethylamine	32	Phenethyldiethylamine	0
Phenethyltrimethylammonium	0	Phenethyltriethylammonium	0
Phenethylbenzylamine	0	Di-(phenethyl)amine	0
β-Hydroxyphenethylamine (phenethanolamine)	22	β-Methoxyphenethylamine	0
β-Ketophenethylamine (ω-aminoacetophenone)	18	β-Ethoxyphenethylamine	0

of this general type was hordenine methochloride (11). The results are shown in Table IV.

Phenylaliphatic Amines As Substrates—Several compounds of the series of phenylalkyl primary carbinamines have been studied by others under various but unrelatable conditions and found to be capable of being oxidized by liver and some other tissue extracts (1, 4, 7, 11, 12). It was desirable to extend the series of ω-phenylalkylamines studied and to include certain derived types, particularly those that have interest because of their con-

siderable physiological activity. The test system was as described for the aliphatic amines and the results (Table V) are therefore placed on a comparable quantitative basis with the data on those compounds as well as with each other.

Series of secondary carbinamines were studied in which an α-alkyl, α-phenyl, or α-phenalkyl group was introduced into the molecule of one of the phenylalkyl primary carbinamines listed in Table V. The conditions of testing were identical with those used for these latter compounds, but in no case was there any oxygen uptake with the following compounds.

α-Methylphenmethylamine (α-phenylethylamine)
α-Methylphenethylamine (phenisopropylamine)
α-Methylphenpropylamine
α-Methylphenbutylamine
α-Methylphenamylamine
α,β-Dimethylphenethylamine (β-methylphenisopropylamine)
α-Methylphenethylmethylamine (phenisopropylmethylamine)
dl,ld-β-Hydroxyphenisopropylamine (*DL*-norephedrine)
dl,ld-β-Hydroxyphenisopropylmethylamine (*DL*-ephedrine)
α-Ethylphenmethylamine (α-phenylpropylamine)
α-Phenylphenmethylamine (benzhydrylamine)
α-Ethylphenethylamine (benzylethylcarbinamine)
α-Propylphenethylamine
α-Isopropylphenethylamine
α-Benzylphenethylamine (dibenzylcarbinamine)
α-Methylphenethylethylamine (phenisopropylethylamine)
dd,ll-β-Hydroxyphenisopropylamine (*DL*-norpseudoephedrine)
dd,ll-β-Hydroxyphenisopropylmethylamine (*DL*-pseudoephedrine)

Two tertiary carbinamines were studied in which two α-methyl groups had been introduced into a couple of the phenylalkyl primary carbinamines previously tested. Testing of these following compounds under the same conditions did not result in any oxygen uptake: α,α-dimethylphenethylamine, α,α-dimethyl-β-hydroxyphenethylamine.

Ring-Substituted Phenylaliphatic Amines As Substrates—The ring-substituted phenethylamines, particularly the phenolic derivatives, have special interest from both biochemical and pharmacological view-points. A few compounds of this type were tested as to their oxidizability by extracts of guinea pig intestine or liver by Blaschko, Richter, and Schlossmann (4). Certain other compounds were later studied (11, 12), but a more systematic study with better quantitative data was needed for any general conclusions as to the effect of any particular type of ring substituent. The effect of the different position of substituents in the ring was also given particular attention. Test conditions were the same as those used for the phenylaliphatic amines. The results are presented in Table VI.

Secondary carbinamines that are α-methyl derivatives of a number of the compounds listed in Table VI were studied to see whether such type compounds, as in the aliphatic and phenylaliphatic series, withstand the

amine oxidase activity under the standard test conditions employed. The following were studied, and all were completely unoxidized by rabbit liver amine oxidase.

3-Hydroxyphenisopropylamine	3-Methylphenisopropylamine
4-Hydroxyphenisopropylamine (paredrine)	4-Methylphenisopropylamine
3-Methoxyphenisopropylamine	3-Methoxy-4-hydroxyphenisopropylamine
4-Methoxyphenisopropylamine (O-methylparedrine)	3-Hydroxy-4-methoxyphenisopropylamine
3,4-Dimethoxyphenisopropylamine	3,4-Dihydroxyphenisopropylamine (hydroxyparedrine)
3,4-Methylenedioxyphenisopropylamine	3,4,5-Trimethoxyphenisopropylamine (α-methylmescaline)
4-Hydroxyphenisopropylmethylamine (N-methylparedrine, paredrinol)	3,4-Dihydroxyphenisopropylmethylamine

Enzyme Constants of Amine Oxidase and Amines—The velocity of the oxidation of the amines with amine oxidase can be expected to follow the well known equation set up by Michaelis and Menten (13) in the form $v = V_{max.}\ (S)/(K_s + (S))$, where the velocity v is dependent on the substrate concentration S and the enzyme-substrate constants K_s and $V_{max.}$ K_s is the kinetic dissociation constant of the enzyme-substrate compound and $V_{max.}$ its oxidation rate at infinite substrate concentration. In the tables, such as Tables II, V, and VI, the relative velocities for the different aliphatic and phenylaliphatic amines at a constant concentration may be expressions of differences either in K_s or $V_{max.}$ or both. A number of experiments were made to value these two constants separately for certain aliphatic and phenylaliphatic type compounds to indicate to what extent they may independently vary.

Substrate concentrations were chosen to make the variation in rate of oxygen uptake between the different Warburg vessels as great as possible, and with concentrations to allow giving a uniform spread for the function $1/S$. The enzyme concentration used was decreased to half that used in the previous experiments with fixed substrate concentration, so that there was but about 0.5 enzyme unit per 2.5 ml. of total volume in the vessel. The data were plotted to conform to the alternate expression of the rate equation offered by Lineweaver and Burk (14) in the form $1/v = K_s/V_{max.}\ (S) + 1/V_{max.}$ where a graph of $1/v$ against $1/S$ intercepts the $1/v$ axis at $1/V_{max.}$ and has the slope $K_s/V_{max.}$ The value of $1/K_s$ is readily obtained from such a graph as the $1/S$ value corresponding to half maximum velocity, which is at $2/V_{max.}$ on the graph, and is indicated in Fig. 3 with light lines. v represents the oxygen uptake per 15 minutes in Fig. 3.

The scattering of points is assumed to be due to experimental errors,

TABLE VI

Maximum Oxidation Rates (Per Cent) Relative to Phenethylamine

At 30° with 0.008 M amine substrates in 0.1 M phosphate, pH 7.0.

2-Hydroxyphenethylamine	43	2-Methylphenethylamine	87
3-Hydroxyphenethylamine	82	3-Methylphenethylamine	68
4-Hydroxyphenethylamine (tyramine)	80	4-Methylphenethylamine	87
2-Methoxyphenethylamine	100	3,4-Dihydroxyphenethylamine (hydroxytyramine)	68
3-Methoxyphenethylamine	98	3-Methoxy-4-hydroxyphenethylamine (homovanillylamine)	22
4-Methoxyphenethylamine (O-methyltyramine)	88	3-Hydroxy-4-methoxyphenethylamine	76
3,4-Dimethoxyphenethylamine	41	3-Methoxy-4,5-methylenedioxyphenethylamine	72
3,4-Methylenedioxyphenethylamine	78	3,4,5-Trimethoxyphenethylamine	0
4-Hydroxyphenethylmethylamine (N-methyltyramine)	70	3,4-Dihydroxyphenethylmethylamine (epinine)	45
4-Hydroxyphenethyldimethylamine (hordenine)	25	3,4,5-Trimethoxyphenethylmethylamine (N-methylmescaline)	0
4-Hydroxyphenethanolmethylamine (synephrine)	24	3,4-Dihydroxyphenethanolmethylamine (*dl*-epinephrine)	10
β-Keto-4-hydroxyphenethylmethylamine (synephrine ketone)	8	β-Keto-3,4-dihydroxyphenethylmethylamine (adrenalone)	5

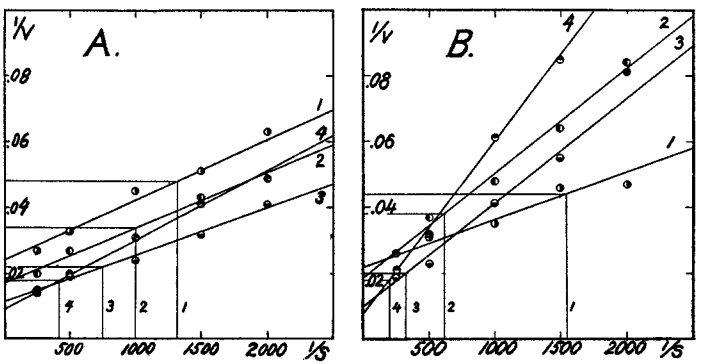

FIG. 3. Reciprocal microliters of oxygen uptake rates against reciprocal molal substrate concentrations at 30° with 2.0 ml. of purified rabbit liver amine oxidase in 0.2 M phosphate buffer, pH 7.0, made up with amine salt solution to a total volume of 2.5 ml. *A*, Curve 1 amylamine, Curve 2 hexylamine, Curve 3 heptylamine, Curve 4 octylamine; *B*, Curve 1 phenethylamine, Curve 2 phenpropylamine, Curve 3 phenbutylamine, Curve 4 phenamylamine.

and not to any non-conformity to the rate equations. In Table VII are presented enzyme constants obtained from the data shown in Fig. 3 and similar data for certain other substrates, all determined with a single enzyme preparation.

The variance in these values is indicated by the finding in later experiments, which were carried out with another enzyme preparation made by the same method but from another rabbit liver, of $1/V_{max.}$ and $1/K_s$ values for phenethylamine of 0.027 and 1800, and for phenethylmethylamine of 0.025 and 3000. In another experiment, the values for phenethylamine were 0.025 and 1360, and in another with 4-hydroxyphenethylamine, the values were 0.031 and 720.

TABLE VII

Constants for Enzyme-Substrate Compounds

Substrate	$\frac{1}{K_s}$	$\frac{1}{V_{max.}}$	K_s	$V_{max.}$
			M	*microliters O_2 per 15 min.*
Amylamine	1320	0.024	0.0008	42
Hexylamine	1000	0.017	0.0010	59
Heptylamine	750	0.011	0.0013	90
Octylamine	420	0.009	0.0024	110
Phenethylamine	1550	0.022	0.00064	45
Phenpropylamine	610	0.019	0.0016	53
Phenbutylamine	320	0.010	0.0031	100
Phenamylamine	200	0.008	0.0050	120
Phenethylamine	1600	0.022	0.00062	45
Phenethylmethylamine	3800	0.026	0.00026	38
4-Hydroxyphenethylamine	670	0.036	0.0015	28
3,4-Dihydroxyphenethylamine	350	0.044	0.0029	23

DISCUSSION

The considerable species variance that is notable from the data of previous workers and those here presented make it questionable that amine oxidase preparations from various sources may be viewed as but a single enzyme. For the present it seems best to keep in mind particular results as being those found under the set of the given experimental conditions. The way that the maximum of activity varies in a particular homologous series with enzyme from different sources is very marked, and may be taken as good evidence of there being different enzymes present. The relatively marked activity of preparations of guinea pig liver upon phenolic and diphenolic amines also would appear to indicate a special character in such respect for the species. That such special characters are a function

of the enzyme itself, rather than due to associated impurities, is indicated by the retention of the relative specificities of rabbit and cattle liver extracts as they are subjected to purification procedures.

While the data given in Tables II through VI show what the relationships of substrate to activity are for the particular set of experimental conditions, the extent to which such data can be taken to indicate expected relationships under other conditions is limited. As shown by the data of Fig. 2, even in a series of compounds having constant dissociation constants, marked variations in activity with pH are seen, and relative activities among a series of compounds are dependent upon the pH of interest.

With regard to relationships between structure and oxidizability by amine oxidase, the differences between primary, secondary, or tertiary carbinamines were most clearly brought out and are of greatest interest because of the importance of this difference in structure for chemical reactivity of the C—N bond. The more expected differences between primary, secondary, and tertiary amines were found to be quite highly dependent upon the structure of the rest of the molecule, and difficult to cover with comprehensive rules. The effect of various substituents in the benzene ring or the side chain of phenethylamines is marked in most cases. The decreased oxidizability of hydroxy and N-methyl compounds is of most interest in connection with their physiological activities.

It should be noted from the data of Table VII that in both aliphatic and phenylaliphatic amines K_s and $V_{max.}$ both increase with chain length of the radical attached to the amino group. The trend of variation of both constants with increasing number of carbon atoms in a homologous series tends to keep the observed rates of oxidation in the series relatively constant when compared at a given substrate concentration. If the rates were determined at relatively high substrate concentrations (corresponding to velocities 4/5 of $V_{max.}$ of the highest $V_{max.}$ of the series), the variation in velocity would be primarily dependent on the variation in $V_{max.}$ and independent of K_s. Under such conditions an increase in the velocity of oxidation would be observed through the series of substrates rather than the passing through a maximum effect in the series as is seen in the experiments of Tables II and V.

The relative velocity of oxidation of these various substrates by amine oxidase by preparations from a liver of a single species is therefore not only dependent on the pH of the determinations, but also on the particular concentration of substrate at which the determination is made. Together, these various factors make it impossible to use oxidation velocities of a series of amines under particular conditions as an exact basis for prediction of relative rates under other conditions of pH and concentration.

Some calculations may be made with respect to the likelihood that amine

oxidase plays a physiological rôle in the inactivation of certain types of amines in the body. By way of example, consider the oxidation rate of tyramine in a concentration of 10^{-5} M with an enzyme concentration of about 0.2 enzyme unit per ml. Substitution in the rate equation $v = V_{max.} (S)/(K_s + (S))$ of 10^{-5} for S and the values of $V_{max.}$ and K_s for tyramine given in Table VII gives v about 0.2 microliter of O_2 per minute, or about 10^{-8} mole of O_2 per minute. On the basis that physiological inactivation would occur with an uptake of 1 mole of O_2 per mole, 10^{-6} mole of tyramine would require about 100 minutes to be inactivated with an enzyme concentration of 0.2 enzyme unit per ml. Such conditions and amounts of tyramine would be approximated in the blood stream of an experimental animal immediately following the intravenous injection of 10^{-6} mole per kilo (0.18 mg. per kilo of hydrochloride) of tyramine. The studies of Clark and Raventos (15) on the relationship between dosage and duration of physiological action of tyramine in cats and man showed that a dosage of 10^{-6} mole per kilo is inactivated in 20 minutes. The liver of the cat contains somewhat more enzyme than that of the rabbit, as shown in Table I, and the over-all concentration in rabbit liver is about 5 enzyme units per gm. Under conditions *in vivo*, the temperature is higher and the pH is somewhat more optimum for the enzyme-tyramine oxidation, according to Hare (1). There is consequently a fair agreement between data *in vitro* and *in vivo*, indicating that indeed the biochemical enzyme studies of amine oxidase may be expected to add much to the physiological perfusion studies of Ewins and Laidlaw (16), and of Guggenheim and Löffler (17) on the destruction of this type of compound in the body.

In passing, it should be pointed out that, although we have not been able to determine the enzyme constants with epinephrine and must leave our studies, it is probable that there will not be a close correspondence of data *in vitro* and *in vivo* with this compound. Our observations do show that $V_{max.}/K_s$ is much lower for this compound than for tyramine, while the *in vivo* data of Clark and Raventos (15) show it to be much more rapidly oxidized than tyramine in the body. Such considerations are in accord with the studies of Richter and Tingey (18) and are more directly confirmed by the establishment of another detoxification pathway for diphenolic amines by Richter (19) and later by Richter and MacIntosh (20).

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